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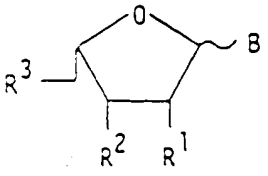
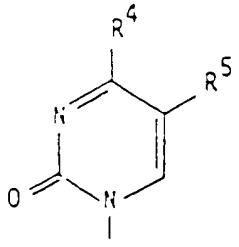
(54) Nucleoside derivatives.

(57) L-ribofuranosyl nucleoside analogues of the formula

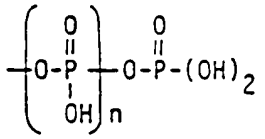


I

wherein B is adenine, guanine, hypoxanthine, 2,6-diaminopurine or



R^1 is H, F; R^3 is H, OH, F, N_3, CN or R^1 and R^2 together constitute a chemical bond;
R^3 is OH or



wherein n is 0, 1 or 2, R^4 is OH, NH_2, R^5 is H, CH_3 or C_2H_5 with certain provisos, in the form of a mixture of alpha and beta anomers or in the form of an alpha or beta anomer for use in therapy in pharmaceutical compositions for therapeutic or prophylactic treatment of infections caused by HIV-viruses, hepatitis B virus or herpes viruses.

EP 0 352 248 A1

Description

Nucleoside derivatives

Field of the invention

The present invention relates to novel chemical compounds and pharmaceutically acceptable salts thereof which can be used in theraphy for therapheutic and prophylactic treatment of the acquired immuno deficiency syndrome (AIDS) and infections caused by viruses requiring reverse transcriptase for replication, such as human immuno deficiency viruses and hepatitis B virus, and also for treatment of other virus diseases, such as those of herpes viruses, diseases which include both common infections and neoplastic diseases, i.e. cancer.

Background of the invention

The effects of viruses on bodily functions is the end result of changes occurring at the cellular and subcellular levels. The pathogenic changes at the cellular level are different for different combinations of viruses and host cells. While some viruses cause a general destruction (killing) of certain cells, other may transform cells into a neoplastic state.

Important common viral infections are herpes dermatitis (including herpes labialis), herpes keratitis, herpes genitalis, herpes zoster, herpes encephalitis, infectious mononucleosis and cytomegalovirus infections all of which are caused by viruses belonging to the herpes virus group. Other important viral diseases are influenza A and B which are caused by influenza A and B virus, respectively. Another important common viral disease is viral hepatitis and especially hepatitis B virus infections are widely spread. Effective and selective antiviral agents are needed for treatment of these diseases as well as for other diseases caused by viruses.

Several different viruses of both DNA and RNA type have been shown to cause tumors in animals. The effect of cancerogenic chemicals can on animals result in activation of latent tumor viruses. It is possible that tumor viruses are involved in human tumors. The most likely human cases known today are leukemias, sarcomas, breast carcinomas, Burkitt lymphomas, nasopharyngeal carcinomas and cervical cancers where RNA tumor viruses and herpes viruses are indicated and papillomas where papilloma viruses are involved. This makes the search for selective inhibitors of tumorogenic viruses and their functions an important undertaking in the efforts to treat cancer.

In the late seventies a new disease was reported, which subsequently was referred to as Acquired Immuno Deficiency Syndrome (AIDS). It is now generally accepted that a retrovirus referred to as HIV (Human Immunodeficiency Virus), formerly known as Human T-cell Lymphotropic Virus (HTLV-III) or Lymphadenopathy Associated Virus (LAV) plays an essential role in the etiology of AIDS. Different types of HIV have been found, such as HIV-1 and HIV-2 and more are likely to be isolated.

AIDS is characterized by a profound immunodeficiency due to low numbers of a subset of lymphocyte-T-helper cells, which are one target for HIV infection. The profound immunodeficiency in AIDS patients makes these patients highly susceptible to a variety of opportunistic infections of bacterial, fungal, protozoal or viral etiology. The etiological agents among viral opportunistic infections are often found in the herpes virus group, i.e. herpes simplex virus (HSV), Varicella Zoster virus (VZV), Epstein-Barr virus (EBV) and, especially, cytomegalovirus (CMV). Other retroviruses affecting humans are HTLV-I and II and examples of retroviruses affecting animals are feline leukemia virus and equine infectious anaemia virus. Human diseases such as multiple sclerosis, psoriasis, tropical spastic paresis and Kawasaki disease have also been reported to be associated with retrovirus infections.

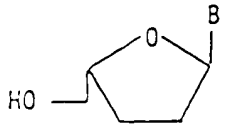
Hepatitis B virus infections cause severe disease such as acute hepatitis, chronic hepatitis, fulminant hepatitis in a considerable number of persons. It is estimated that there are 200 million patients with chronic hepatitis B infection in the world. A considerable number of the chronic cases progress to liver cirrosis and liver tumours. In some cases the hepatitis infections also take a rapid and severe course as in fulminant B hepatitis with about 90 % mortality. At present there is no known effective treatment against hepatitis B infections. The replication of hepatitis B virus is similar to that of retroviruses and it contains the same essential virus reverse transcriptase activity.

Prior art

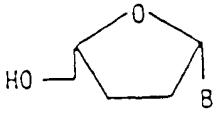
A great number of nucleoside analogues exhibit several antimetabolic activities. They do so by substituting for or competing with the naturally occuring nucleosides. Recently some nucleoside analogues have been described, which inhibit in cell culture the multiplication of human immunodeficiency virus (HIV, also called HTLV-III, LAV) the causative agent of AIDS and AIDS-related complex (ARC). The naturally occuring nucleosides and most nucleoside analogues described which inhibit HIV multiplication are beta anomers where the sugar ribofuranose has the D-configuration.

The synthesis of the compound β-2'-deoxy-L-uridine has been described by A. Holy in Nucleic Acid Chemistry Vol. 1 (1978) pp 347-353 (Eds L. B. Townsend and R. S. Stuart, Wiley, New York N.Y.) and by A. Holy in Coll. Czech. Chem. Commun. Vol. 37 (1972) pp 4072-4087. In this latter publication the syntheses of β-2'-deoxy-L-thymidine and β-2'-deoxy-L-cytosine also are described.

EP-A2-0 285 884 describes a process to produce α- and β-L-2',3'-dideoxy-nucleosides and their use as antiviral and antiobiotic agents. Said compounds can be represented by the formulas



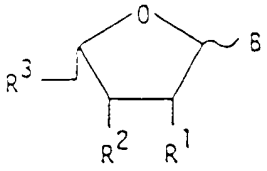
and



wherein B can be adenine, guanine, hypoxanthine, diaminopurine, uracil, cytosine, thymine and 5-ethyluracil

Disclosure of the invention

The present invention relates to new L-ribofuranosyl nucleoside analogues of the formula I



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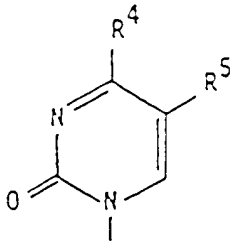
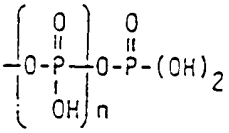
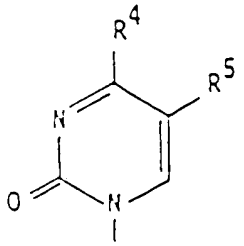
wherein B is adenine, guanine, hypoxanthine, 2,6-diaminopurine or



and the radicals R^1, R^2, R^3, R^4 and R^5 are defined as follows:
R^1: H, F;
R^2: H, OH, F, N_3, CN or R^1 and R^2 together constitute a chemical bond ;
R^3: OH or

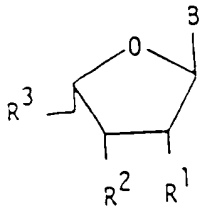


wherein n = 0, 1 or 2 ;
R^4: OH, NH_2 ;
R^5: H, CH_3, C_2H_5 ;
with the provisos that when R^1 is H and R^3 is OH, then R^2 must not be H, and further that when in the β-anomer R^1 is H, R^2 is OH and R^3 is OH, B is adenine, guanine, hypoxanthine, 2,6-diaminopurine or

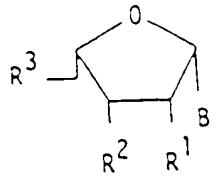


wherein R^5 is C_2H_5 when R^4 is OH and R^5 is CH_3 or C_2H_5 when R^4 is NH_2, and pharmaceutically acceptable salts thereof.

The compounds of the formula I may have the alpha- or the beta-configuration. According to the Freudenberg convention (1932) the same configuration at the anomeric center (C-1 for aldoses) and the last asymmetric centre (C-4 for pentoses) are termed alpha-anomers. Thus the compounds of the formula I have the configuration Ia (alpha) and Ib (beta)



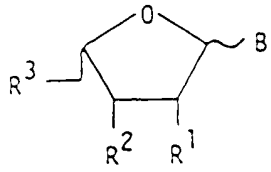
Ia (alpha)



Ib (beta) ;

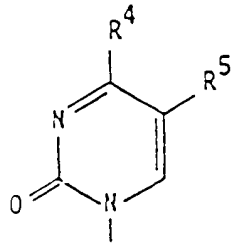
Said compounds have been found to inhibit the multiplication of human immunodeficiency virus (HIV)

The invention also refers to compounds of the formula I



I

wherein B is adenine, guanine, hypoxanthine, 2,6-diaminopurine or

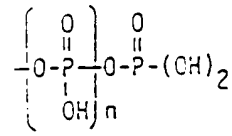


and the radicals R^1, R^2, R^3, R^4 and R^5 are defined as follows:

R^1: H, F;

R^2: H, OH, F, N_3, CN or R^1 and R^2 together constitute a chemical bond ;

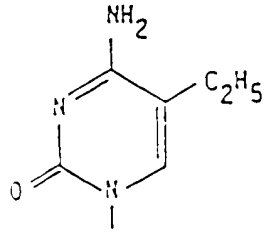
R^3: OH or



wherein n = 0, 1 or 2 ;

R^4: OH, NH_2 ;

R^5: H, CH_3, C_2H_5; with the proviso that when R^1 is H, R^2 is H and R^3 is OH B is



in the form of a mixture of alpha and beta anomers or in the form of an alpha or beta anomer; and pharmaceutically acceptable salts thereof, for use in therapy.

The compounds of the formula I are useful as therapeutic and/or prophylactic agents in the control and treatment of HIV virus infections in man. In a more general aspect, the compounds of the formula I are useful as therapeutic and, or prophylactic agents in the control and treatment of infections caused by retroviruses and hepatitis B virus in mammals and man.

All retroviruses, including HIV, require the enzyme reverse transcriptase in their natural cycle of replication.

Hepatitis B virus (HBV) is a DNA virus with a unique circular double-stranded DNA genome which is partly single-stranded. It contains a specific DNA polymerase required for viral replication. This DNA polymerase also acts as a reverse transcriptase during the replication of HBV DNA via an RNA intermediate.

The compounds of the formula I inhibit the activity of reverse transcriptase of retroviruses including HIV.

A possible area of use for the compounds of the formula I is for treatment of infections caused by hepatitis B virus.

Another possible area of use for the compounds of the formula I is in the treatment of herpes virus infections. Among the herpes viruses may be mentioned Herpes simplex type 1 and 2, varicella (Herpes zoster), virus causing infectious mononucleosis (i.e. Epstein-Barr virus), cytomegalovirus and human herpes virus type 6. Important diseases caused by herpes viruses are herpes dermatitis (including herpes labialis), herpes genitalis, herpes keratitis, herpes encephalitis and herpes zoster.

Another possible area of use for the compounds of the present invention is in the treatment of cancer and tumors, particularly those caused by viruses. This effect may be obtained in different ways, i.e. by inhibiting the transformation of virus-infected cells to a neoplastic state, by inhibiting the spread of viruses from transformed cells to other normal cells and by arresting the growth of virus-transformed cells.

The invention furthermore provides:

A pharmaceutical composition comprising a compound of the formula I as an active ingredient and a pharmaceutically acceptable carrier, including lipsomes; and

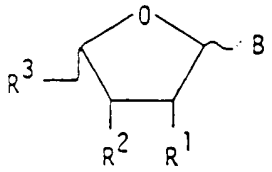
A method for therapeutic and/or prophylactic treatment of virus infections in an animal or human host in need of treatment comprising administering an effective amount of a compound of the formula I.

It is a preferred aspect of the invention to treat infections caused by viruses requiring reverse transcriptase for replication, including human immuno deficiency viruses and hepatitis B virus.

The invention also relates to the use of a compound of the formula I for the manufacture of a medicament for therapeutic and/or prophylactic treatment of the acquired immuno deficiency syndrome and infections caused by viruses requiring reverse transcriptase for replication.

Preferably they can be used for the treatment of infections caused by HIV viruses or hepatitis B virus.

Preferred compounds of the formula I



I

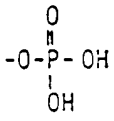
are those wherein

R^1 is H, F

R^2 is H, F, N_3

R^1 and R^2 together constitute a chemical bond

R^3 is OH or



i.e. a monophosphate ester thereof

R^4 is OH, NH_2

R^5 is H, CH_3

Examples of especially preferred compounds are those of the formula I wherein

R^1	R^2	R^3	R^4	R^5
H	F	OH	NH_2	H
H	F	OH	NH_2	CH_3
H	F	OH	OH	CH_3
H	N_3	OH	OH	CH_3
———		OH	NH_2	H
———		OH	OH	CH_3
H	H	OPO_3H_2	NH_2	H
H	H	OPO_3H_2	NH_2	CH_3
H	H	OPO_3H_2	OH	CH_3

Examples of pharmaceutically acceptable salts of the compounds of formula I include base salts, e.g. derived from an appropriate base, such as alkali metal (e.g. sodium, potassium, alkaline earth metal, e.g. magnesium) salts, ammonium and NX_4^+ (wherein X is C_{1-4} alkyl). Physiologically acceptable acid salts include salts of organic carboxylic acids such as acetic, lactic, gluconic, citric, tartaric, maleic, malic, pantothenic, isethionic, oxalic, lactobionic and succinic acids; organic sulfonic acids such as methanesulfonic, ethanesulfonic, benzenesulfonic, p-chlorobenzenesulfonic and p-toluenesulfonic acids and inorganic acids such as hydrochloric, hydroiodic, sulfuric, phosphoric and sulfamic acids.

Mono-, di- and triphosphate esters of the compounds are also included in the invention. Physiologically acceptable counterions of the phosphate groups include inorganic and organic counterions. Inorganic counterions are for example ammonium, sodium, potassium, lithium, magnesium and calcium. Organic counterions are derived from non-toxic bases, such as primary, secondary and tertiary amines, including naturally occuring amines. Examples of such amines are diethylamine, triethylamine, isopropylamine, ethanolamine, morpholine, 2-diethylaminoethanol, glucosamine, N-methylglucamine, piperazine and dicyclohexylamine.

In clinical practice the nucleoside analogues of the formula I will normally be administered orally, by injection or by infusion in the form of a pharmaceutical preparation comprising the active ingredient in the form of the original compound or optionally in the form of a pharmaceutically acceptable salt thereof, in association with a pharmaceutically acceptable carrier which may be a solid, semi-solid or liquid diluent or an ingestible capsule. The compound may also be used without carrier material. As examples of pharmaceutical preparations may be mentioned tablets, dragées, capsules, granulates, suspensions, elixirs, syrups, solutions, liposomes etc. Usually the active substance will comprise between 0.05 and 20 % for preparations intended for injection and between 10 and 90 % for preparations intended for oral adminsitration.

In the treatment of patients suffering from retrovirus, especially HIV, or hepatitis B virus infections, it will be preferred to administer the compounds by any suitable route including the oral, parenteral, rectal, nasal, topical and vaginal route. The parenteral route includes subcutaneous, intramuscular, intravenous and sublingual administration. The topical route includes buccal and sublingual administration. The dosage at which the active ingredients are administered may vary within a wide range and will depend on various factors such as the severity of the infection, the age of the patient etc., and may have to be individually adjusted. As a possible range for the amount of the compounds of the invention or a physiologically acceptable salt thereof to be administered per day may be mentioned from about 10 mg to about 10 000 mg, preferentially 100-500 mg for intravenous administration and preferentially 100-3000 mg for oral administration.

Compounds of the formula I can cooperate synergistically or additively with a wide range of other therapeutic agents, thereby enhancing the therapeutic potential of both agents without adding the toxic effects, thus increasing the therapeutic ratio.

Therefore, a compound of formula I or a pharmaceutically acceptable derivative thereof can be used in combination therapy, wherein the two active agents are present in a ratio resulting in an optimal therapeutic ratio. This can be provided either by a synergistic effect against the viral infection and/or by a decrease in toxicity while maintaining a therapeutic effect which is additive or synergistic.

The optimal therapeutic ratio is observed when the two agents are present in a ratio of 500:1 to 1:500, preferably 100:1 to 1:100, particularly 20:1 to 1:20 and especially 10:1 to 1:10.

Said combination may conveniently be administered together, for example, in a unitary pharmaceutical formulation, or separately for example as a combination of tablets and injections administered at the same time or at different times, in order to achieve the required therapeutic effect.

The compounds of the formula I are potentiated by interferons, other antiviral agents such as foscarnet, AZT, fluorothymidine, HIV protease inhibitors, immunomodulators, intereron inducers and growth factors.

Particularly preferred types of interferon are α, β and γ interferon inducers such as "Ampligen" (Hem

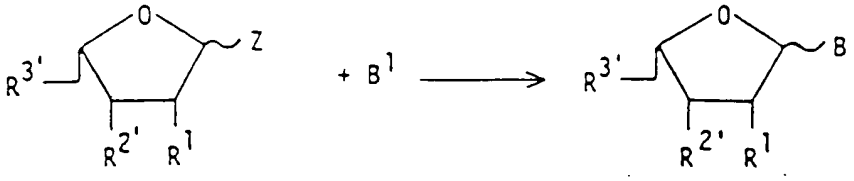
Research).

Other combinations suitable for use according to the present invention include those wherein the second agent is, for example, interleukin II, suramin, foscarnet esters, HPA 23, inhibitors of HIV protease such as pepstatin, steroids, medications such as levamisol or thymosin to increase lymphocyte numbers and/or function as appropriate, or GM-CSF and other factors regulating cell functions

Methods of preparation

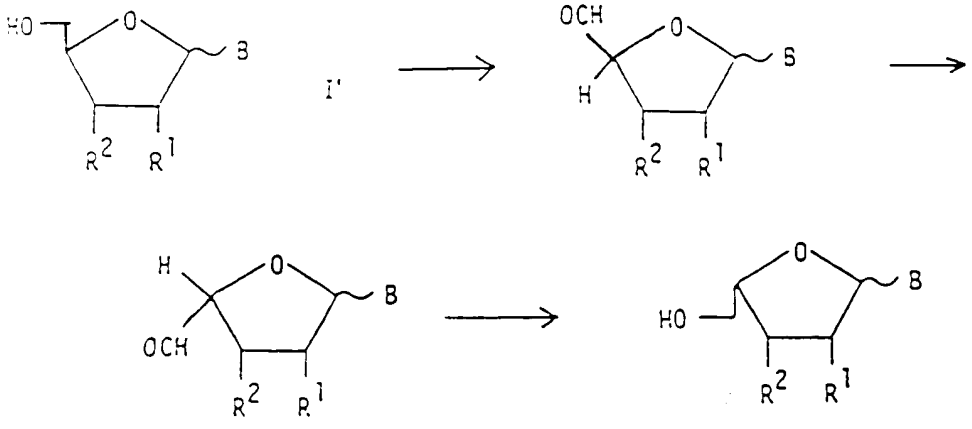
The compounds of the invention may be prepared by general methods, constituting a further aspect of the invention.

Condensing a glycoside as comprised in formula I where the hydroxyl groups may be optionally protected to the N-1 position of a purine or pyrimidine derivative, according to known methods described in the literature. Such methods are described for example in "Basic Principles in Nucleic Acid Chemistry", Vol. 1 (Academic Press, 1974, Ed. P.O.P.Ts'o), in "Nucleoside analogues, Chemistry, Biology and Medical Applications" (Pharma Press, 1979, Eds. R.T. Walker, E. De Clercq and F. Eckstein).



Examples of suitable derivatives of the reacting species are those wherein Z is Cl, Br, I, acyloxy or alkoxy, R^2 and R^3 is R^2 and R^3 respectively as defined above with the proviso that when R^2 or R^3 is OH said OH must be protected as O-acyl, O-benzoyl, O-benzyl or O-silyl (e.g. dimethyl, tert-butylsilyl). The bases B^1 may be protected with a silyl protecting group such as $(CH_3)_3Si$ and/or with alkyl och acyl protecting groups. The acyl protecting groups may be used on amino groups of B and alkyl protecting groups (etherderivatives) may be used on carbonyl oxygens of the bases B. After condensation the products may by hydrolyzed or converted by conventional methods, known to those skilled in the art, into compounds of the formula I.

The compounds may also be prepared by a general method whereby the 5'-hydroxymethyl function of a corresponding nucleoside analogue, having the ordidary D-configuration of the ribose moiety, is oxidized to an aldehyde functional group and the stereochemistry at the 4'-position is inverted or racemized, after which the aldehyde group is reduced to a 5'-hydroxymethyl function and the nucleoside analogue having the L-configuration is isolated from the reaction products.



wherein B, R^1 and R^2 are defined as above.

This reaction sequence has been described for example by J.G. Moffat in Nucleoside Analogues page 88 ff, 1979 (Plenum Press, Eds. R.T. Walker, E. Declercq and F. Eckstein).

Another method for syntheses of various 2 or 3 substituted L-sugar derivatives is to prepare a 2,3-anhydro-L-ribofuranoside or 2,3-anhydro-L-lyxofuranoside analogous to what has been described for the synthesis of 2,3-anhydro-D-ribofuranoside and 2,3-anhydro-D-lyxofuranoside by for example M. Taniguchi et al in Chem. Pharm. Bull. Volume 22, pages 2318 to 2323,, 1974. The 2,3-epoxides would then be reacted with a nucleophile to introduce a fluorine, azido or other functional group and the newly created hydroxylic functional group could be reduced to a hydrogen atom. Finally the so created L-furanoside analogue would be condensed with a purine or pyrimidine base B whereby all the reactions in the sequence are carried out by methods known to those skilled in the arts.

The following examples will further illustrate the invention:

Example 1. 1-(2,3-Dideoxy-alpha,beta-L-ribofuranosyl)-cytosine

1-(2,3-Dideoxy-5-O-tert-butyldiphenylsilyl-alpha, beta-L-ribofuranosyl)-cytosine (170 mg) was dissolved in 2 M sodium hydroxide (5 ml, ethanol-water 1:1) and stirred at ambient temperature for 3 days. Thin layer chromatography (TLC, silica, ethylacetate-methanol 4:1) shows 2 spots, Rf 0.2 and Rf 0.8, for the unprotected title compound and for the free silyl protecting group respectively. Dowex 50Wx8 [pyridinium]$^+$ (2 g) was added, the solution was filtered and the solvent was evaporated in vacuo. The residue was triturated with diethyl ether, dissolved in methanol and filtered through a cotton plug. The solvent was evaporated to give as a residue 1-(2,3-dideoxy-alpha,beta-L-ribofuranosyl)-cytosine (52 mg). The product was a mixture of two anomers in an approximate ratio of 1:6.

^{13}C NMR (Jeol FX 200, DMSO-d6)δ: 163.5(d, C-2, α, β); 152.4 (s, C-4); 142.3(d, C-5, α, β); 93.4(d, C-6, α, β); 86.9(d, C-1', α, β); 82.0(d, C-4', α, β); 63.0(d, C-5', α, β); 32.0(d, C-2', α, β); 25.5(d, C-3', α,β).

Example 2. 9-(2,3-Dideoxy-alpha,beta-L-ribofuranosyl)-adenine

9-(2,3-Dideoxy-5-O-tert-butyldiphenylsilyl-alpha,beta-L-ribofuranosyl)-adenine (218 mg) was dissolved in 1 M tetrabutylammoniumfluoride in tetrahydrofuran (1 ml) and stirred at ambient temperature for 1 hour. Thin layer chromatography (TLC, silica, ethyl acetate-methanol 5:1) shows 2 spots Rf 0.25 and Rf 0.8, for the unprotected title compound and for the free silyl protecting group respectively. The solvent was evaporated and the crude product was purified by chromatography on a small column of silica (Merck, Kieselgel 60) eluated with ethylacetate-methanol 4:1, followed by purification on a reverse phase plate, RP8, to give 9-(2,3-dideoxy-alpha,beta-L-ribofuranosyl)-adenine (8 mg). The product was a mixture of two anomers in approximately equal amounts.

^{13}C NMR (Jeol FX200, DMSO-d6)δ: 153.9(s,); 141.6(s); 141.3(s); 87.5(d, C-i', α, β); 83.4(d, C-4', α, β); 65.0(d, C-5', α, β); 33.5(d, C-2', α, β); 25.6(d, C-3', α, β). Mass spectrum (Jeol DX-300/DA 5000, FAB: 6 kV Xe atoms): 235.1055; $C_{10}H_{14}N_5O_2$ mass 235.1069.

The starting materials for the two compounds in examples 1 and 2 were prepared by the following sequence of reactions a-c:

a) 1-Acetyl-2,3-dideoxy-5-O-tert-butyldiphenylsilyl-alpha, beta-L-ribofuranoside

R-γ-tert-Butyldiphenylsilyloxymethyl-γ-butyrolactone (14 g) in dry diethyl ether (300 ml) was cooled to -78° C and stirred while diisobutylaluminium hydride in hexane (65 ml, 1.1 M) was added over a period of 30 min. Methanol (15 ml) was added and the reaction solution was slowly warming to room temperature. The solution was extracted with aqueous sodium hydrogencarbonate, dried over magnesium sulfate, and the solvent was evaporated. The residue was dissolved in dry pyridine, acetic anhydride (about 3 equivalents) was added and the reaction solution was heated at 60° C for 4 hours. By TLC (silica, ethylacetate-hexane 1:4) a new spot appears, corresponding to the reaction product, Rf 0.5. The solvent was evaporated in vacuo, the residue was dissolved in diethyl ether, and the new solution was extracted with aqueous sodium hydrogencarborate and dried (sodium sulfate). The solvent was evaporated to give as a residue 1-acetyl-2,3-dideoxy-5-O-tert-butyldiphenylsilyl-alpha,beta-L-ribofuranoside (8.8 g).

^{13}C NMR (Jeol FX 200, $CDCl_3$)δ: 99(d, C-1, α, β); 82(d, C-4, α, β); 66(d, C-5, α, β); 32(d, C-2, α, β); 27(s, CH_3, tert-butyl); 25(d, C-3, α, β); 22(s, CH_3); 18(s, C, tert-butyl).

b) 1-(2,3-Dideoxy-5-O-tert-butyldiphenylsilyl-alpha,beta-L-ribofuranosyl)-cytosine

Cytosine (0.3 g) in hexamethyldisilazane (4 ml), acetonitrile (4 ml) and chloro trimethylsilane (0.1 ml) under an athmosphere of nitrogen was heated at reflux until a clear solution had formed (about 15 min). The solvent was evaporated in vacuo (1 mm, 40° C) and 1-acetyl-2,3-dideoxy-5-O-tert-butyldiphenylsilyl-alpha,beta-L-ribofuranoside (1.0 g) dissolved in acetonitrile (10 ml) was added. The solution was cooled (0° C) and $SnCl_4$ (0.29 ml) in acetonitrile (5 ml) was added during 1 min. The cooling-bath was removed and the reaction solution was stirred at ambient temperature for 2 hours. By TLL (silica, ethylacetate-methanol, 5:1) a new spot with Rf 0.25 appears, corresponding to the reaction product. Methanol (2 ml) and aqueous ammonia (25 %, 2 ml) were added. The solvent was evaporated to dryness, the residue was dissolved in ethyl acetate, filtered and the solvent was evaporated. Purification by chromatography on silica (Kiselgel Merck 60, 15 % methanol in ethyl acetate) afforded 1-(2,3-dideoxy-5-O-tert-butyldiphenylsilyl-alpha,beta-L-ribofuranosyl)-cytosine (340 mg) as a pure product.

^{13}C NMR (Jeol FX 200, CD_3OD)δ: 165.8(s, C-4); 156.2(s, C-2); 140.0(d, C-5, α, β); 94.1(d, C-6, α, β); 87(d, C-1', α, β); 81.6(d, C-4' α, β); 65.0(d, C-5', α, β); 33(d, C-2', α, β); 26.4(s, CH_3, tert-butyl); 25(d, C-3', α, β); 18.8(s, C, tert-butyl).

c) 9-(2,3-Dideoxy-5-O-tert-butyldiphenylsilyl-alpha,beta-L-ribofuranosyl)-adenine

The title compound was prepared analogous to the synthesis of the corresponding cytosine derivative. 1-(2,3-dideoxy-5-O-tertbutyldiphenylsilyl-alpha,beta-L-ribofuranosyl)-cytosine. The amounts of the various reagents are as follows: adenine (373 mg) heated in hexamethyldisilazane (4 ml), acetonitrile (2.5 ml) and chloro trimethylsilane (0.3 ml). Addition of 1-acetyl-2,3-dideoxy-5-O-tert-butyldiphenylsilyl-alpha,beta-L-ribofuranoside (1.0 g) in acetonitrile (5 ml) cooled to -5° C. $SnCl_4$ (0.29 ml) in acetonitrile (5 ml).

The product 9-(2,3-dideoxy-5-O-tert-butyldiphenylsilyl-alpha,beta-L-ribofuranosyl)-adenine (218 mg) was purified by chromatography on silica (10% methanol in ethyl acetate).
TLC (silica, ethyl acetate-methanol, 9:1) Rf 0.3 ^{13}C NMR (Jeol FX 200, CD_3OD)δ: 153.1(s, C-2) 141.0(s, C-8), 135-125 (SiPh); 87.2 (d, C-1', α, β); 83.2(d, C-4', α, β). 67.0(d, C-5', α, β), 33.6(d, C-2', α, β). 27.6(s, CH3, tert-butyl), 26.0(d, C-3', α, β); 20.3(s, C, tert-butyl).

Example 3. 1-(2,3-Dideoxy-3-fluoro-α-L-ribofuranosyl)-thymine

To a chilled solution of 2',3'-dideoxy-3'-fluoro-thymidine (108 mg, 0.3 mmol) in CH_2Cl_2 (20 ml) at -78°C, DMSO (118 mg, 1.5 mmol) and $(CF_3CO)_2O$ (315 mg, 1.5 mmol) was added. After 15 min, the reaction was warmed up to room temperature and stirred for an additional period of 2 h. Methanol was added to quench the reaction. Volatile matters were removed in vacuo. The residue was redissolved in dry tetrahydrofurane at room temperature and K-tert butoxide (1.1 g, 10 mmol) was added and stirred at 45°C for 2 h under N_2. The reaction mixture was then neutralized by addition of 50 % ethanolic-acetic acid. Volatile matters were removed completely by co-evaporation with dry toluene. The residue was redissolved in ethanol (20 ml) and $NaBH_4$ (756 mg, 20 mmol) added and stirred at 50° C to give the title compound in a mixture of different products.

Example 4. 1-(2,3-Dideoxy-3-fluoro-β-L-ribofuranosyl)-thymine

The compound is prepared from 1-(2,3-dideoxy-3-fluoro-α-D-ribofuranosyl)-thymine by a procedure analogous to what has been described in example 3.

This compound can also be prepared starting from 1-(2-deoxy- α -D-threopentofuranosyl)-thymine, which first is oxidized to the 5'-aldehyde and isomerized and then reduced to 1-(2-deoxy-β-L-threo-pentofuranosyl)thymine, analogous to what has been described in Example 3. After protection of the 5'-hydroxyl group, the 3'-hydroxyl group is reacted with diethylamino sulfurtrifluoride (DAST) whereby a fluorine atom is introduced with inversion of configuration and the title compound is formed.

By analogous procedures 1-(2,3-dideoxy-3-azido-β-L-ribofuranosyl)-thymine can also be prepared from 1-(2,3-dideoxy-3-azido-α-D-threopentofuranosyl)thymine, and 1-(2,3-dideoxy-3-fluoro/azido-α-L-ribofuranosyl)thymine can be prepared from 1-(2,3-dideoxy-3-fluoro/azido-β-D-threo-pentofuranosyl)thymine.

Analogously 1-(2,3-dideoxy-3-azido-α-L-ribofuranosyl)-thymine and 1-(2,3-dideoxy-3-azido-β-L-ribofuranosyl)-thymine can also be prepared from their corresponding α- and β-D-ribofuranosyl thymine derivatives respectively.

Biological tests

Test I Effect of compounds of the formula I on HIV in H9 cells

Materials and methods: HIV infection of H9 cells

H9 cells, 10^5 cells per well on a 24 well plate, suspended in 2 ml RPMI-medium containing 10 % fetal calf serum, 100 µg/ml penicillin, 10 µg/ml streptomycin sulfate and 2 µg/ml polybrene are exposed to HIV (HTLV-III_B) and different concentrations of the test compounds. The plates are incubated at 37°C in 5 % CO_2 for 6-7 days. The contents in each well is then homogenized with a pipette and transferred to a centrifuge tube. After centrifugation for 10 min at 1500 rpm the supernatant is removed and the cell pellet is analyzed by fixing in methanol on glass plates. Human HIV positive serum diluted 1:80 or 1:160 is added and incubated for 30 min at 37°C. The plate is then washed with phosphate-buffered saline (PBS) containing Ca^{2+} and Mg^{2+}. Sheep anti-human conjugate (FITC) is added and after a new incubation the plate is again washed with PBS. Contrast straining is done with Evans blue and after drying the frequency of HIV antigen containing cells is determined in a microscope. The test result is shown in Table 1.

Table 1.

Concentration (µM) for 50 % inhibition (IC_{50}) of human immuno deficiency virus multiplication in cell culture

Compound (code)	IC_{50} µM
1-(2,3-Dideoxy-alpha,beta-L-ribofuranosyl)-cytosine (VSB 815)	< 1

Table 1 shows that the tested compound is an active inhibitor of HIV virus multiplication.

Test II Cellular toxicity

H9 cells, $2x10^7$ cells per plate, are incubated in RPMI-1640 medium containing 10 % fetal calf serum, 70 mg/l penicillin, 100 mg/l streptomycin and 10 mM hepes, in absence or presence of test compounds. The number of cells per plate is determined after 48 h. Cells incubated in the absence of test compounds then underwent two

cell division cycles.

F5000 cells, which are human embryo cells, $1x10^5$ cells per plate, are incubated in Eagle's minimal essential medium, supplemented with Earle's salts, non-essential amino acids, 10 % fetal calf serum, 10 mM hepes, 70 mg/l penicillin and 100 mg/l streptomycin, in absence or presence of test compounds. The number of cells per plate is determined after 48 h. Cells incubated in the absence of test compounds underwent one cell division cycle. The results are given as % inhibition of cell multiplication when the concentration of the compound is 100 µM or 250 µM. The test results are given in table 2.

Table 2.

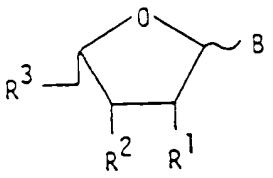
Cellular toxity on H9 and F5000 cells

Compound (code)	% Inhibition (Conc. µM) H9	F5000
1-(2,3-Dideoxy-alpha,beta-L-ribofuranosyl)-cytosine (VSA 815)	35 (200)	55 (100)

Table 2 shows that the concentrations at which the compound exhibit toxicity exceed the concentration needed to 50 % inhibition of HIV multiplication as given in table 1.

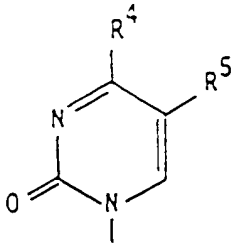
Claims

1. A compound of the formula

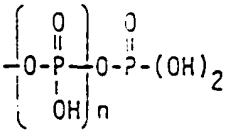


I

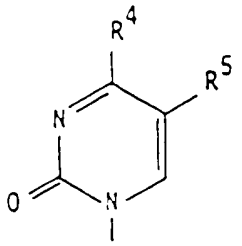
wherein B is adenine, guanine, hypoxanthine, 2,6-diaminopurine or



and the radicals R^1, R^2, R^3, R^4 and R^5 are defined as follows:
R^1: H, F;
R^2: H, OH, F, N_3, CN or R^1 and R^2 together constitute a chemical bond ;
R^3: OH or

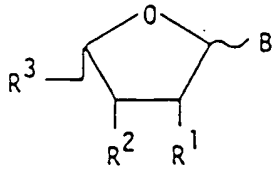


wherein n = 0, 1 or 2 ;
R^4: OH, NH_2
R^5: H, CH_3, C_2H_5 ;
with the provisos that when R^1 is H and R^3 is OH, then R^2 must not be H, and further that when in the β-anomer R^1 is H, R^2 is OH and R^3 is OH, B is adenine, guanine, hypoxanthine, 2,6-diaminopurine or



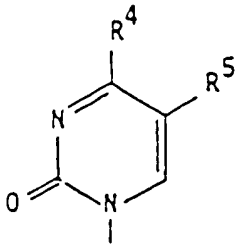
wherein R^5 is C_2H_5 when R^4 is OH and R^5 is CH_3 or C_2H_5 when R^4 is NH_2, in the form of a mixture of alpha and beta anomers or in the form of an alpha or beta anomer; and pharmaceutically acceptable salts thereof.

2. A compound according to claim 1, wherein R^3 is OH.
3. A compound according to any of claims 1-2, wherein R^1 and R^2 together constitute a chemical bond.
4. A compound according to any of claims 1-2, wherein R^1 is H and R^2 is F or N_3.
5. A compound according to any of claims 1-4, wherein R^4 is NH_2 and R^5 is H or CH_3.
6. A compound according to any of claims 1-4, wherein R^4 is OH and R^5 is H or CH_3.
7. A compound of the formula

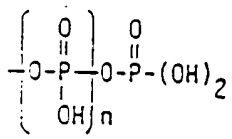


I

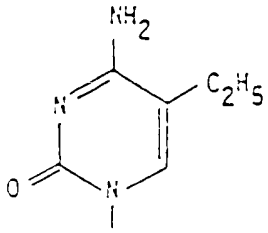
wherein B is adenine, guanine, hypoxanthine, 2,6-diaminopurine or



and the radicals R^1, R^2, R^3, R^4 and R^5 are defined as follows:
R^1: H, F;
R^2: H, OH, F, N_3, CN or R^1 and R^2 together constitute a chemical bond ;
R^3: OH or



wherein n = 0, 1 or 2 ;
R^4: OH, NH_2 ;
R^5: H, CH_3, C_2H_5; with the proviso that when R^1 is H, R^2 is H and R^3 is OH B is



in the form of a mixture of alpha and beta anomers or in the form of an alpha or beta anomer, and pharmaceutically acceptable salts thereof, for use in therapy.

8. A compound of the formula I according to any of claims 1-6 for use in therapy.

9. A pharmaceutical composition comprising as an active ingredient a compound of the formula I according to any of claims 1-7 and a pharmaceutically acceptable carrier, including liposomes.

10. A method for therapeutic and/or prophylactic treatment of virus infections in an animal or human host in need of treatment, comprising administering an effective amount of a compound of the formula I as defined in any of claims 1-7.

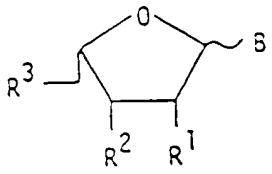
11. A method according to claim 10 for treatment of infections caused by viruses requiring reverse transcriptase for replication, including human immuno deficiency virus and hepatitis B virus.

12. A method according to claim 10 for treatment of infections caused by herpes viruses.

13. Use of a compound of the formula I according to any of claims 1-7 for the manufacture of a medicament for therapeutic and/or prophylactic treatment of the acquired immuno deficiency syndrome and infections caused by viruses requiring reverse transcriptase for replication.

14. Use according to claim 13 for the treatment of infections caused by HIV-viruses, hepatitis B virus, or herpes viruses.

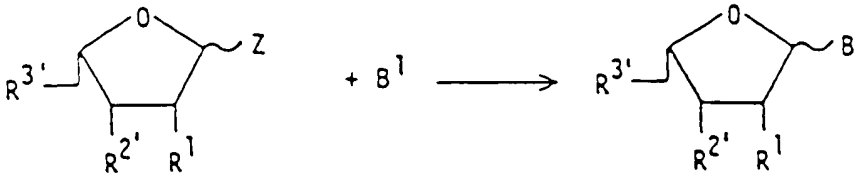
15. A process for preparation of a compound of the formula



I

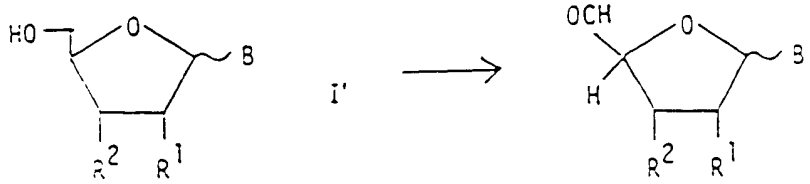
wherein B, R^1, R^2 and R^3 are as defined in claim 1, by

a) condensing a glycoside as comprised in the formula I to the N-1 position of a pyrimidine derivative or to the N-9 position of a purine derivative



wherein Z is Cl, Br, J, acyloxy or alkoxy, $R^{2'}$ and $R^{3'}$ are R^2 and R^3, respectively, as defined above or with the proviso that when R^2 or R^3 is OH then O must have a protecting group, B^1 is B as defined above having a silyl, acyl or alkyl protecting group; or

b) oxidizing the 5'-hydroxymethyl function of a D-ribofuranosyl nucleoside analogue of the formula I' to an aldehyde

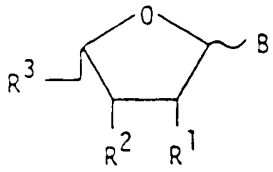


I'

wherein B, R^1 and R^2 are as defined above, inverting the configuration at the 4'-position, isolating the L-stereomer and reducing it to the L-ribofuranosyl nucleoside analogue of the formula I.

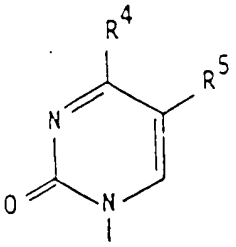
Claims for the following Contracting States: GR,ES

1. A process for preparation of a compound of the formula

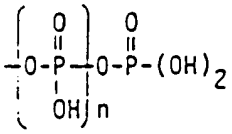


I

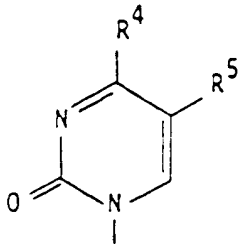
wherein B is adenine, guanine, hypoxanthine, 2,6-diaminopurine or



and the radicals R^1, R^2, R^3, R^4 and R^5 are defined as follows:
R^1: H, F;
R^2: H, OH, F, N_3, CN or R^1 and R^2 together constitute a chemical bond ;
R^3: OH or

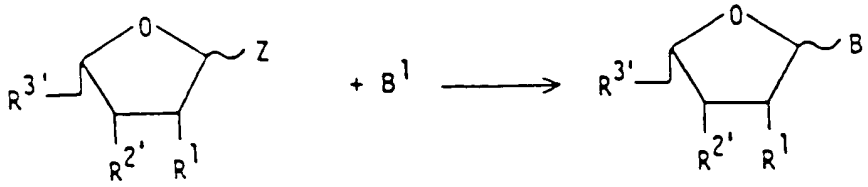


wherein n = 0, 1 or 2 ;
R^4: OH, NH_2 ;
R^5: H, CH_3, C_2H_5 ;
with the provisos that when R^1 is H and R^3 is OH, then R^2 must not be H, and further that when in the β-anomer R^1 is H, R^2 is OH and R^3 is OH, B is adenine, guanine, hypoxanthine, 2,6-diaminopurine or



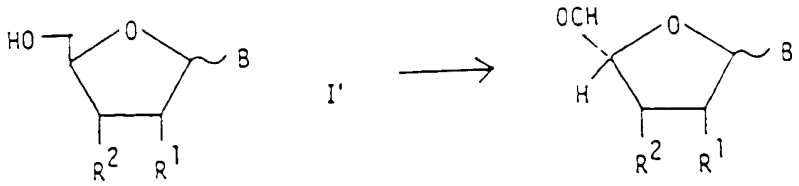
wherein R^5 is C_2H_5 when R^4 is OH and R^5 is CH_3 or C_2H_5 when R^4 is NH_2, by

a) condensing a glycoside as comprised in the formula I to the N-1 position of a pyrimidine derivative or to the N-9 position of a purine derivative



wherein Z is Cl, Br, J, acyloxy or alkoxy, $R^{2'}$ and $R^{3'}$ are R^2 and R^3, respectively, as defined above or with the proviso that when R^2 or R^3 is OH then O must have a protecting group, B^1 is B as defined above having a silyl, acyl or alkyl protecting group; or

b) oxidizing the 5'-hydroxymethyl function of a D-ribofuranosyl nucleoside analogue of the formula I' to an aldehyde



wherein B, R^1 and R^2 are as defined above, inverting the configuration at the 4'-position, isolating the L-stereomer and reducing it to the L-ribofuranosyl nucleoside analogue of the formula I.

2. A process according to claim 1, wherein R^3 is OH.

3. A process according to any of claims 1-2, wherein R^1 and R^2 together constitute a chemical bond.

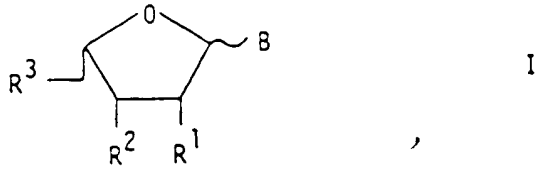
4. A process according to any of claims 1-2, wherein R^1 is H and R^2 is F or N_3.

5. A process according to any of claims 1-4, wherein R^4 is NH_2 and R^5 is H or CH_3.

6. A process according to any of claims 1-4, wherein R^4 is OH and R^5 is H or CH_3.

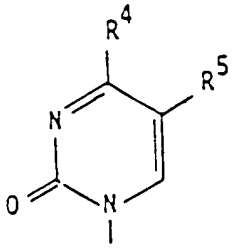
7. A process according to any of claims 1-6, wherein the compound of the formula I is obtained in the form of a mixture of alpha and beta anomers or in the form of an alpha or beta anomer and optionally is converted into a pharmaceutically acceptable salt.

8. Use of a compound of the formula



I

wherein B is adenine, guanine, hypoxanthine, 2,6-diaminopurine or

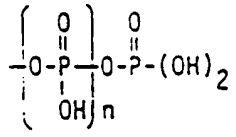


and the radicals R^1, R^2, R^3, R^4 and R^5 are defined as follows:

R^1: H, F;

R^2: H, OH, F, N_3, CN or R^1 and R^2 together constitute a chemical bond ;

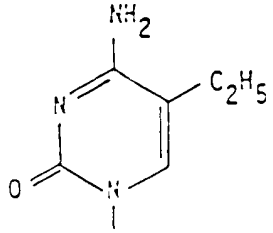
R^3: OH or



wherein n = 0, 1 or 2 ;

R^4: OH, NH_2 ;

R^5: H, CH_3, C_2H_5; with the proviso that when R^1 is H, R^2 is H and R^3 is OH B is



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Mechanism of Action and In Vitro Activity of 1′,3′-Dioxolanylpurine Nucleoside Analogues against Sensitive and Drug-Resistant Human Immunodeficiency Virus Type 1 Variants

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(−)-β-D-1′,3′-Dioxolane guanosine (DXG) and 2,6-diaminopurine (DAPD) dioxolanyl nucleoside analogues have been reported to be potent inhibitors of human immunodeficiency virus type 1 (HIV-1). We have recently conducted experiments to more fully characterize their in vitro anti-HIV-1 profiles. Antiviral assays performed in cell culture systems determined that DXG had 50% effective concentrations of 0.046 and 0.085 μM when evaluated against HIV-1_{IIIB} in cord blood mononuclear cells and MT-2 cells, respectively. These values indicate that DXG is approximately equipotent to 2′,3′-dideoxy-3′-thiacytidine (3TC) but 5- to 10-fold less potent than 3′-azido-2′,3′-dideoxythymidine (AZT) in the two cell systems tested. At the same time, DAPD was approximately 5- to 20-fold less active than DXG in the anti-HIV-1 assays. When recombinant or clinical variants of HIV-1 were used to assess the efficacy of the purine nucleoside analogues against drug-resistant HIV-1, it was observed that AZT-resistant virus remained sensitive to DXG and DAPD. Virus harboring a mutation(s) which conferred decreased sensitivity to 3TC, 2′,3′-dideoxyinosine, and 2′,3′-dideoxycytidine, such as a 65R, 74V, or 184V mutation in the viral reverse transcriptase (RT), exhibited a two- to fivefold-decreased susceptibility to DXG or DAPD. When nonnucleoside RT inhibitor-resistant and protease inhibitor-resistant viruses were tested, no change in virus sensitivity to DXG or DAPD was observed. In vitro drug combination assays indicated that DXG had synergistic antiviral effects when used in combination with AZT, 3TC, or nevirapine. In cellular toxicity analyses, DXG and DAPD had 50% cytotoxic concentrations of greater than 500 μM when tested in peripheral blood mononuclear cells and a variety of human tumor and normal cell lines. The triphosphate form of DXG competed with the natural nucleotide substrates and acted as a chain terminator of the nascent DNA. These data suggest that DXG triphosphate may be the active intracellular metabolite, consistent with the mechanism by which other nucleoside analogues inhibit HIV-1 replication. Our results suggest that the use of DXG and DAPD as therapeutic agents for HIV-1 infection should be explored.

Reverse transcriptase (RT) inhibitors play a cornerstone role in the therapy for human immunodeficiency virus type 1 (HIV-1) infection. Based on structure and mechanism of action, these inhibitors can be classified into two major groups, nucleoside RT inhibitors (NRTIs) and nonnucleoside RT inhibitors (NNRTIs). NRTIs are usually 2′,3′-dideoxy derivatives of natural substrates of DNA polymerases. All NRTIs are believed to act in a similar fashion to inhibit the RT activity (9, 15, 20, 34, 39); i.e., following intracellular conversion to their 5′-triphosphate derivatives, they bind to RT in competition with natural substrates and subsequently cause chain termination through incorporation into the nascent DNA strand. Chain termination is caused by the lack of a 3′-hydroxyl motif, which is needed to form a 3′-5′ phosphodiester bond with the next nucleoside substrate in the elongating DNA strand. NNRTIs are a group of compounds which specifically inhibit HIV-1 RT by binding to a hydrophobic pocket close to the polymerase active site, which results in a direct inactivation of RT (8, 14, 18, 26, 33, 38).

Currently, the appearance of drug-resistant virus is an inevitable consequence of prolonged exposure of HIV-1 to antiretroviral agents. This is believed to be caused by both a high turnover of HIV-1 in patients (16, 37) and low fidelity of the viral RT (7). To achieve efficient inhibition of HIV-1 replication in patients, and to delay or prevent the appearance of drug-resistant virus, drug combinations have been used effectively in treating HIV-1 infection (2, 22). However, recent studies have suggested that HIV-1 can become multidrug resistant under combination therapy, albeit requiring a longer time to develop than in a single-drug regime (4, 31). Therefore, it is still necessary to develop alternate drug combinations for the long-term successful treatment of HIV-1 infection. We are focusing our efforts on developing new agents which are effective against existing drug-resistant virus and can be rationally incorporated into combination drug therapy.

In this regard, several groups have recently reported the synthesis of pyrimidine- and purine-derived nucleoside analogues containing a dioxolane sugar derivative, in which an oxygen atom is found at the 3′ position of the sugar ring (3, 6, 21). The β-D analogues of purine bases have been reported to be potent anti-HIV-1 agents (17, 32). In particular, (−)-β-D-1′,3′-dioxolane guanosine (DXG) and (−)-β-D-2,6-diaminopurine dioxolane (DAPD) have been reported to inhibit HIV-1 in vitro (17, 32). Following oral administration of DAPD to woodchucks or rhesus monkeys, plasma concentrations of DXG are significantly higher than those of DAPD (23, 24). These data suggest that DAPD is quickly converted into DXG in vivo and should be considered a prodrug of DXG. None of the currently approved anti-HIV-1 nucleoside analogues con-

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tains a dioxolane sugar motif. Therefore, DXG and its putative prodrug analogue represent a novel class of nucleosides with potential utility in anti-HIV-1 therapy. The present report describes our results in further elucidating the mechanism of action of DXG and DAPD. In addition, we have examined the effect of these compounds on wild-type (wt) and drug-resistant clinical isolates of HIV-1, alone and in combination with other NRTIs or NNRTIs.

MATERIALS AND METHODS

Reagents. DXG, DAPD, DXG 5′-triphosphate (DXG-TP), (+)-β-D-1′,3′-dioxolane guanosine, and 2′,3′-dideoxy-3′-thiacytidine (3TC) were synthesized at BioChem Pharma as previously described (3, 32). All of the dioxolanyl nucleosides were enantiomerically pure. 3′-Azido-2′,3′-dideoxythymidine (AZT) and 2′,3′-dideoxycytidine (ddC) were purchased from Sigma (Oakville, Ontario, Canada). Ultrapure nucleoside 5′-triphosphates, 2′-deoxynucleoside 5′-triphosphates (dNTPs), 2′,3′-dideoxynucleoside 5′-triphosphates (ddNTPs), and polynucleotides poly(rA) · oligo(dT)$_{12-18}$ and poly(rC) · oligo(dG)$_{12-18}$ were purchased from Pharmacia Biotech Inc. (Montreal, Quebec, Canada). AZT triphosphate (AZT-TP) and 3TC triphosphate (3TC-TP) were purchased from Moravek Biochemicals. [^{3}H]dGTP, [^{3}H]TTP, and [γ-^{32}P]ATP were obtained from Du Pont NEN (Montreal, Quebec, Canada). [^{3}H]thymidine was obtained from Amersham (Oakville, Ontario, Canada). Nevirapine was generously provided by Boehringer-Ingelheim Inc. (Burlington, Ontario, Canada).

Cells and viruses. Human cord blood mononuclear cells (CBMCs) and peripheral blood mononuclear cells (PBMCs) were obtained from HIV-1-negative and hepatitis B virus-negative donors (Department of Obstetrics, Jewish General Hospital, Montreal, Quebec, Canada) and isolated by Ficoll-Hypaque (Pharmacia) density gradient centrifugation. Cells were cultured in RPMI 1640 medium (Gibco BRL Laboratories, Mississauga, Ontario, Canada) containing 0.1% (vol/vol) (5 μg/ml) phytohemagglutinin (Boehringer Mannheim, Montreal, Quebec, Canada), 10% fetal calf serum (Flow Laboratories, Toronto, Ontario, Canada), 2 mM glutamine, 100 U of penicillin per ml, 100 μg of streptomycin per ml, and 15 U of interleukin 2 (Boehringer Mannheim) per ml. Cells were incubated at 37°C, in an atmosphere of 5% CO_2, for 3 to 4 days prior to being used for antiviral assays (27).

The T-cell lines MT-2, MT-4, H9, and Jurkat were obtained from either the National Institutes of Health AIDS Research and Reference Reagents (Rockville, Md.) or the American Type Culture Collection (Manassas, Va.). These cells were used for antiviral and cytotoxicity studies and were maintained as suspension cultures in RPMI 1640 medium containing 10% fetal calf serum, 2 mM glutamine, 100 U of penicillin per ml, and 100 μg of streptomycin per ml. Other tumor cell lines, Molt-4, HT-1080, DU145, and HepG2, were also obtained from the American Type Culture Collection. One normal cell line, human skin fibroblasts (HSF), was obtained from M. Chevrette (McGill University, Montreal, Quebec, Canada). These cells were used for cytotoxicity assays. HSF, HT-1080, DU145, and HepG-2 cells were cultured in minimal essential medium. Molt-4 cells were cultured in RPMI 1640 medium.

HIV-1$_{IIIB}$ and the recombinant HIV-1 clone HXB2-D were kindly supplied by R. C. Gallo (Institute of Human Virology, Baltimore, Md.). Recombinant mutated HIV-1 variants were prepared by site-directed mutagenesis as previously described (10, 12). HIV-1 clinical isolates were obtained by coculture of peripheral blood lymphocytes from HIV-1-infected individuals with CBMCs and then propagated on CBMCs in the absence of drugs as previously described (28).

Antiviral assays. The anti-HIV-1 activities of DXG and DAPD were assessed by employing HIV-1$_{IIIB}$ in a variety of cell types as previously described (10, 12, 25, 28). A number of recombinant drug-resistant variants and low-passage clinical isolates from individuals who had received long-term anti-HIV therapy were also used to evaluate the effects of these two compounds. Briefly, cells were incubated for 2 to 3 h with virus at a multiplicity of infection of 0.005 for T-cell assays or 0.5 for monocytic-cell assays. The infected cells were cultured in the presence of the test compound for 5 to 7 days. The anti-HIV-1 efficacy was determined by testing for HIV-1 RT activity in the cell culture supernatants. All assays were performed in duplicate, and at least two independent experiments were performed. AZT and/or 3TC was used as a control in each experiment. The data are expressed as the means of the 50% effective concentrations (EC_{50}s) as calculated from the linear portion of the dose-response curve.

Effects of combining DXG and standard anti-HIV-1 agents were assessed in CBMCs by using HIV-1$_{IIIB}$. The combinations were performed by using a checker board cross pattern of drug concentrations. The antiviral effects were determined by monitoring RT activity in the culture supernatants at day 7. The data were analyzed according to the method described by Chou and Talalay (5). The combination indices (CIs) of DXG with other anti-HIV-1 agents were calculated by using CalcuSyn software (Biosoft, Cambridge, United Kingdom). Theoretically, a CI value of 1 indicates an additive effect, a CI value of >1 indicates antagonism, and a CI value of <1 indicates synergism.

Cytotoxicity analysis. Cellular toxicity was assessed by [^{3}H]thymidine uptake and WST-1 staining. In the [^{3}H]thymidine uptake experiments, Molt-4, HT1080, DU-145, HepG2, and HSF were plated at a concentration of 1×10^5 to 2×10^5 cells per well (96-well plates). Phytohemagglutinin-stimulated PBMCs were cultured at a concentration of 4×10^6 per well. Following a 24-h preincubation period, test compounds (at 10^{-4} to 10^{-10} M concentrations) were added and the cells were incubated for an additional 72 h. [^{3}H]thymidine was added during the final 18-h incubation period. The cells were then washed once with phosphate-buffered saline, treated with trypsin if the cells were adherent, and resuspended in water (hypotonic lysing of cells). The cellular extract was applied directly to a Tomtec Harvester 96 apparatus. The 50% cytotoxic concentration (CC_{50}) was determined by comparing the radioactive counts per minute obtained from drug-tested samples to those obtained from the control (untreated) cells.

In the WST-1 staining experiments, cell lines were cultured in RPMI medium in 96-well plates at a density of 5×10^4 cells/well. CBMCs were plated at a concentration of 0.5×10^6/well. Compounds (at 10^{-4} to 10^{-7} M concentrations) were added at day zero. Cell viability was assessed on day 7 by using the WST-1 reagent (Boehringer Mannheim) in accordance with the protocol provided by the supplier.

RT inhibition assay. wt recombinant HIV-1 RT was expressed as a histidine-tagged protein in *Escherichia coli* and purified to 95% homogeneity as previously described (11, 13). Inhibition of HIV-1 RT RNA-dependent DNA polymerase activity by DXG-TP was assessed by employing both homopolymeric and heteropolymeric RNA templates/DNA primers (T/P). The heteropolymeric RNA template (HIV-PBS)/18-mer oligodeoxynucleotide primer (dPR) was prepared as described previously (11). The reverse transcription reaction mixture contained final concentrations of 50 mM Tris-HCl (pH 7.8), 60 mM KCl, 10 mM $MgCl_2$, 0.1 U of homopolymeric T/P per ml, 5 μM dNTP substrate or 25 nM HIV-PBS/dPR, and 5 μM each dATP, dCTP, dGTP, and dTTP in 100 μl. Reaction mixtures were incubated for 30 min at 37°C in the presence or absence of ddNTP inhibitors as described previously (11).

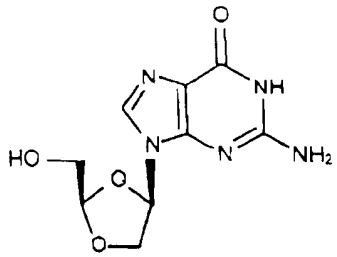
The effect of DXG-TP on RT activity was also assessed by using a chain termination/dNTP incorporation assay in which inhibition of nascent DNA synthesis (chain termination) was monitored based on cDNA synthesis as previously described (1, 13).

Determination of HIV-1 RT genotype. To determine the RT genotypes of the HIV-1 clinical isolates, proviral DNA of each isolate was extracted from infected CD4$^+$ T cells or CBMCs and the complete RT coding regions were amplified by PCR as previously reported (10). The PCR product was purified and then directly sequenced by using primer RTS (5′-CCAAAAGTTAAACAATGGC-3′), which corresponds to the 5′ portion of the RT coding region (nucleotides 2603 to 2621 of HXB2-D coordinates).

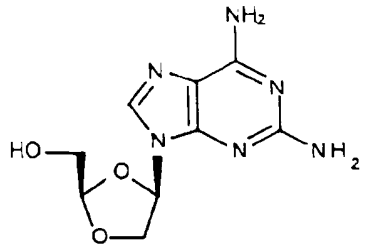
RESULTS

Inhibition of HIV-1 RT polymerase activity by DXG-TP. The chemical structures of 1′,3′-dioxolanylpurine nucleosides DXG and DAPD are shown in Fig. 1. DXG-TP is the active antiviral form of DAPD in vivo (23, 24). To better define the molecular mechanism by which these nucleoside analogues inhibit HIV-1, DXG was chemically converted to its triphosphate derivative (DXG-TP) and tested for its direct effect on HIV-1 RT. The inhibitory effect of DXG-TP on HIV-1 RT activity was assessed by using various homopolymeric and heteropolymeric T/P (Table 1). DXG-TP was a potent HIV-1 RT inhibitor, with a 50% inhibitory concentration (IC_{50}) of 0.012 μM, when using wt HIV-1 RT, complementary T/P poly(rC) · oligo(dG), and substrate dGTP (Table 1). This value is similar to that obtained for ddGTP. Similarly, DXG-TP and ddGTP were observed to have the same inhibitory effect on HIV-1 RT when the heteropolymeric T/P (HIV-PBS/dPR) was used (Table 1). The inhibition of HIV-1 RT by DXG-TP was observed to occur via competition with the natural substrate; i.e., the higher the concentration of dGTP, the lower the inhibitory effect of DXG-TP (data not shown). In addition, as expected, DXG-TP did not show any inhibition of HIV-1 RT activity at concentrations up to 10 μM when the noncomplementary T/P poly(rA) · oligo(dT) was used along with dTTP as the substrate (Table 1).

We also analyzed the effect of DXG-TP on HIV-1 RT activity by a chain elongation/termination assay which provides a method to directly visualize the incorporation of dideoxynucleotide monophosphates into nascent DNA by monitoring the reaction products through polyacrylamide gel electrophoresis. It was showed that DXG monophosphate was incorporated into the nascent DNA strands and resulted in chain termination (results not shown). The pattern of chain termination



(-)-β-D-1',3'-dioxolane guanosine (DXG)



(-)-β-D-2,6-diaminopurine dioxolane (DAPD)

FIG. 1. Molecular structures of dioxolanylpurines.

generated by incorporation of DXG-TP into elongating DNA strands was exactly the same as that for ddGMP. In general, the inhibitory effect of DXG-TP on RT activity in this cell-free assay was approximately equivalent to those observed for ddGTP and AZT-TP but stronger than the chain termination observed for 3TC-TP (results not shown).

Cellular toxicity. DXG and DAPD, along with 3TC and AZT, were also tested for their effect on cell proliferation by measuring [^{3}H]thymidine uptake and cell toxicity by WST-1 cell viability assays. DXG and DAPD did not inhibit cell proliferation at concentrations up to 500 μM in various cells (Table 2). In the same experiments, CC_{50}s for AZT and ddC were found to be less than 10 μM. DXG was not toxic to human CBMCs (Table 2), MT-2, H9, or Jurkat cell lines (data not shown) at concentrations up to 100 μM by a WST-1 cell viability assay. In contrast, the CC_{50}s obtained for AZT and ddC in CBMCs were 74 and 29 μM, respectively (Table 2).

Anti-HIV-1 efficacy in different cell types. The anabolism of nucleoside analogues can be greatly influenced by cell type. Therefore, we assessed the anti-HIV-1 activity of DXG and DAPD in human CBMCs and a variety of human T-cell lines. A dose-response curve showing the inhibition of HIV-1_{IIIB} in MT-2 cells is presented in Fig. 2. The results indicate that the activity of DXG is approximately equivalent to that of 3TC but is 5- to 10-fold lower than that of AZT. DAPD was approximately 10-fold less active than DXG. The EC_{50}s obtained for the test compounds in primary cells and cell lines are compiled in Table 3.

We also compared the antiviral activities of DXG and (+)-β-D-1',3'-dioxolane guanosine. Our results show that the (+) enantiomer (EC_{50}, 0.7 μM) has less activity against HIV-1_{IIIB} in MT-2 cells than the (−) enantiomer (EC_{50}, 0.085 μM).

Susceptibility of recombinant drug-resistant HIV-1 variants. Recombinant HIV-1 variants carrying a drug resistance mutation(s) were employed to test the cross-resistance phenotypes of DXG and DAPD in CBMCs and MT-2 cells. All of the recombinant viral strains were derived from HXB2-D. A summary of the genetic backgrounds of the variants and their sensitivities to the dioxolanylpurine compounds, 3TC and AZT, in CBMCs is shown in Table 4. These viruses contain mutations seen for the most common RT inhibitor- and protease inhibitor-resistant HIV-1 variants (summarized in reference 29). The variants of HIV-1 carrying 2',3'-dideoxyinosine (ddI), ddC, or 3TC resistance mutations (i.e., 65K, 74V, and 184V substitutions, respectively) in the RT gene had minimally (two- to fivefold) decreased sensitivity to DXG and DAPD compared to the wt HXB2-D in CBMCs (Table 4). Similar results were obtained when the recombinant viruses were tested in MT-2 cells (data not shown). In addition, the variant bearing mutations 41L, 215Y, and 184V had approximately a twofold-decreased sensitivity to DXG, which was similar to that of the 184V single-mutant recombinant. This variant had high-level resistance to 3TC but increased sensitivity to AZT (36).

In contrast, AZT-resistant virus, carrying multiple substitutions (41L, 70R, 215Y, and 219Q) in its RT gene, remained completely sensitive to DXG and DAPD in CBMCs (Table 4) and MT-2 cells (data not shown). In addition, the dioxolanyl nucleoside analogues were effective against NNRTI-resistant (EC_{50} = 0.05 μM) and protease inhibitor-resistant (EC_{50} = 0.12 to 1.37 μM) variants (Table 4).

Susceptibility of HIV-1 clinical isolates. The sensitivity of viruses found in clinical isolates to antiviral chemotherapy might be quite variable due to the presence of quasispecies. In addition, HIV-1 isolates obtained from patients receiving long-term antiretroviral therapy might behave differently from cloned viruses containing genetically engineered mutations in the RT gene. For these reasons, clinical isolates of HIV-1 from antiviral-agent-naive and drug-treated patients were assayed in CBMCs for their sensitivity to DXG and DAPD. A summary of the recent therapy histories of the patients from which the HIV-1 isolates were obtained, the RT genotypes of the isolates, and their sensitivities to the various anti-HIV agents is presented in Table 5.

TABLE 1. Inhibition of HIV-1 RT by DXG-TP and other ddNTPs

Template · primer	Substrate	IC_{50} (μM) of:		
		DXG-TP	ddGTP	ddTTP
poly(rC) · oligo(dG)$_{12-18}$	dGTP	0.012 ± 0.002	0.011 ± 0.0007	ND[a]
poly(rA) · oligo(dT)$_{12-18}$	dTTP	>10[b]	ND	0.024 ± 0.003
HIV-PBS/dPR	dNTPs[c]	0.062 ± 0.007	0.074 ± 0.008	ND

[a] ND, not determined.
[b] The highest concentration of inhibitor used in the inhibition study was 10 μM.
[c] Each dNTP (dATP, dCTP, dGTP, and dTTP) was at 5 μM.

TABLE 2. Effect of nucleoside analogues on cell proliferation

Cells	[^{3}H]thymidine uptake [CC_{50} (μM)][a,c]				
	DXG	DAPD	3TC	AZT	ddC
PBMC	>500	>500	>500	9	35.5
Molt-4	>500	>500	ND[b]	3	2
HT-1080	>500	>500	>500	5	2
HepG2	>500	≥500	350	3	7
DU145	>500	>500	>500	>10	5
HSF	≥500	>500	400	>10	2
CBMC[c]	>100	>100	ND	74	29

[a] The highest concentrations of DXG and DAPD used in these studies were 500 μM in [^{3}H]thymidine uptake and 100 μM in WST-1 staining.
[b] ND, not determined.
[c] CC_{50}s for CBMC were obtained by WST-1 staining.

TABLE 3. Inhibitory effects of nucleoside analogues on HIV-1 replication[a]

Cells	EC_{50} (μM)[b] of:			
	DXG	DAPD	3TC	AZT
CBMC	0.046 ± 0.017	0.97 ± 0.092	0.023 ± 0.011	0.0051 ± 0.003
MT-2	0.085 ± 0.026	0.54 ± 0.29	0.091 ± 0.08	0.0076 ± 0.0044
MT-4	0.051	0.94	0.056	0.008
Jurkat	0.34	1.37	0.53	0.011
H9	0.06	0.075	ND[c]	0.041

[a] All assays were performed with laboratory strain HIV-1_{IIIB}.
[b] The values presented are the means ± standard deviations of data from at least three experiments performed in duplicate. Values without standard deviations are the averages of data from two independent experiments conducted in duplicate.
[c] ND, not determined.

Four isolates (no. 3887, 4246, 4877, and 4526) were sensitive to AZT and/or 3TC or had marginally decreased sensitivity to one of these two drugs compared with recombinant variants (Table 4). Using these four isolates, the EC_{50}s obtained for both DXG and DAPD (Table 5) were comparable to those observed with the wt strains HIV-1_{IIIB} and HXB2-D assessed in CBMCs (Tables 3 and 4).

Isolates 3350 and 4205, obtained from patients who had received 3TC therapy and carried the 184V mutation, were 3TC resistant and AZT sensitive. These 184V mutant isolates had an approximately fivefold-decreased susceptibility to DXG compared to the 3TC- and AZT-sensitive isolates (Table 5). This was consistent with the results obtained with the recombinant variants (Table 4).

Clinical isolate 4242, obtained from a patient treated with AZT, exhibited decreased sensitivity to AZT but remained sensitive to DXG, DAPD, and 3TC. The NNRTI-resistant strain 4924, isolated from an individual undergoing AZT therapy, had an EC_{50} for nevirapine of >10 μM but was sensitive to the dioxolane nucleoside analogues (Table 5). The protease inhibitor-resistant isolate 4833 was obtained from an individual who had received 48 weeks of saquinavir therapy. This isolate exhibited a 20-fold-decreased sensitivity (EC_{50} = 0.11 μM) to the protease inhibitor compared with the baseline isolate, 4526 (EC_{50} = 0.0063 μM). The 4833 isolate remained sensitive to the dioxolanyl compounds (EC_{50} = 0.17 μM for DXG and 0.63 μM for DAPD) (Table 5).

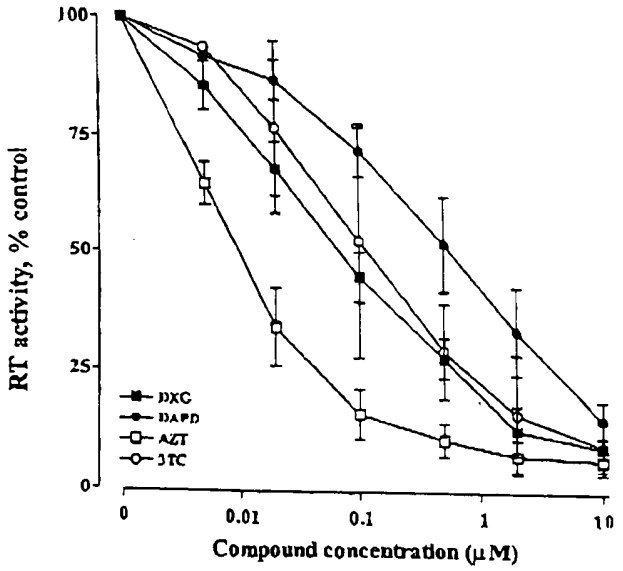


FIG. 2. Dose-response curve of inhibition of HIV-1 replication. MT-2 cells were infected with HIV-1_{IIIB} at a multiplicity of infection of 0.005. The infected cells were cultured in the presence of various concentrations of antiviral compounds as indicated. Viral susceptibility to the compounds was assayed by measurement of HIV-1 RT activity in the culture supernatants as described in Materials and Methods. Data are expressed as means ± standard deviations of data from at least five separate experiments, each performed in duplicate.

Anti-HIV drug combination effects. The antiviral efficacy of DXG against HIV-1_{IIIB} was assessed in combination with AZT, 3TC, or nevirapine in CBMCs. The CIs of DXG in combination with the approved anti-HIV-1 agents are summarized in Table 6. The CIs were calculated at several different effective concentrations (EC_{50}, EC_{75}, and EC_{90}) and in different molar ratios of the combined drugs. The CIs obtained were between 0.4 and 0.9 in the case of DXG combined with either 3TC or nevirapine, which suggests that DXG had moderate synergy with these two agents. However, DXG demonstrated greater synergy with the thymidine analogue AZT, with CIs between 0.3 to 0.8 at the EC_{50} and less than 0.3 at higher effective concentrations, which indicates a strong synergy.

DISCUSSION

The dioxolanyl guanosine analogues DXG and DAPD were previously reported to possess anti-HIV and anti-hepatitis B virus activities (17, 30, 32). In this article, we present an expanded and detailed evaluation of antiviral and biochemical characteristics of DXG and DAPD.

In vitro antiviral assays demonstrated that both DXG and DAPD had very promising anti-HIV-1 activity in the various types of cells tested. Comparison of the (−) and (+) enantiomers of β-1′,3′-dioxolane guanosine showed that both were effective inhibitors of HIV-1, but the (−) enantiomer, DXG, displayed approximately 10-fold higher activity. Generally, the anti-HIV-1 activity of DXG was at the same level as that observed for 3TC in our assays but was 5- to 10-fold less than that of AZT. In vivo, DAPD is quickly and efficiently converted into DXG after either oral or intravenous administration to woodchucks or rhesus monkeys (23, 24). This biotransformation is believed to be a deamination process catalyzed by a ubiquitous enzyme, adenosine deaminase. In our experiments, DAPD had consistently lower anti-HIV activity than DXG. These results were consistent with previous reports for the antiretroviral effects of these two dioxolanylpurine nucleoside analogues (17, 32). The relatively low anti-HIV-1 activity of the putative prodrug, DAPD, in cell culture may reflect less-effi-

TABLE 4. Effect of DXG and DAPD on recombinant drug-resistant strains of HIV-1

Recombinant variant[a]	Resistant to	EC_{50} (μM) in CBMCs[b]			
		DXG	DAPD	3TC	AZT
HXB2-D (wt)		0.21	1.1	0.041	0.0041
65R	ddI, ddC, 3TC, PMEA[g]	1.2	6.5	0.36	0.003
74V	ddI	1.3	6.6	0.12	0.006
184V	3TC, ddI, ddC	0.44	2.1	>50[c]	0.0027
41L 70R 215Y 219Q	AZT	0.24	1.25	0.062	0.082
41L 215Y 184V	3TC	0.41	2.3	>50[c]	0.006
106A 181C[d]	NNRTIs	0.05	ND[e]	ND	0.03
10R 46I 63P 82T 84V[f]	Protease inhibitors	0.12	1.37	ND	0.0032

[a] The recombinant viruses were wt and mutants harboring the indicated substitution(s) in the RT.
[b] Values are the averages of data from two independent experiments performed in duplicate.
[c] The highest concentration of 3TC used in these assays was 50 μM.
[d] The EC_{50} for nevirapine was >10 μM.
[e] ND, not determined.
[f] Protease genotype; the EC_{50} for saquinavir was 0.075 μM.
[g] PMEA, 9-(2-phosphonylmethoxyethyl)adenine.

cient metabolic conversion into the active form, DXG. This does not necessarily indicate that this compound has less anti-HIV-1 activity in vivo. From a pharmaceutical point of view, DAPD might be equipotent to DXG in vivo. The ultimate choice of compound to advance into human therapy would be dependent on other parameters, such as oral bioavailability (23, 24).

The appearance of drug-resistant virus following prolonged administration of antiviral agents is a major obstacle for the therapy of HIV-1 infection. Therefore, we conducted antiviral assays to elucidate the cross-resistance profiles of the novel dioxolanylpurine analogues. Using recombinant and clinical drug-resistant HIV-1 variants carrying the most common RT inhibitor resistance-related mutations, it was demonstrated that DXG and DAPD possess marginal cross-resistance with 3TC (Tables 4 and 5). Our results show that HIV-1 strains carrying 65R and 74V substitutions in their RT genes had approximately fivefold-reduced sensitivity to the dioxolane compounds, which is consistent with previously reported values (19). However, both DXG and the DAPD retained their potency to AZT-resistant, NNRTI-resistant, and protease inhibitor-resistant HIV-1 variants. These data suggest that DXG or DAPD could be used as an alternative drug for the treatment of HIV-1-infected individuals who have developed tolerance to currently approved drug regimens.

Combination therapy has proven to be an exciting approach to combat HIV-1 infection. Combination therapy increases the therapeutic efficiency of anti-HIV agents and delays or prevents selection of drug-resistant virus. Combination profiles with approved anti-HIV-1 agents have become an essential prerequisite for new drug candidates. DXG showed synergy with the NRTIs AZT and 3TC and the NNRTI nevirapine (Table 6). These data suggest that the combination of DXG with approved anti-HIV agents in the treatment of HIV-1 infection could have increased clinical benefits. Previous reports have demonstrated that the mutations 65R, 74V, and 184V in HIV-1 RT, which confer minimal cross-resistance to DXG and DAPD, can revert AZT-resistant virus to AZT sensitivity (19, 35, 36). The strong in vitro synergy of DXG and AZT, combined with the increased sensitivity of the AZT drug resistance phenotype, indicates a potential benefit for a DXG and AZT combination.

The guanosine analogue dideoxyguanosine displays excellent anti-HIV activity in vitro. However, it was never developed into an antiretroviral agent because of its high cellular toxicity. DXG and DAPD had relatively low cellular toxicity to human primary cells and a variety of established normal and tumor cell lines. Neither compound showed significant inhibition of cellular DNA synthesis or cell proliferation, and both were found to be much less toxic than AZT or ddC (Table 2).

TABLE 5. Susceptibility of HIV-1 isolates from patients treated with nucleoside analogues to DXG and DAPD

Viral isolate	Antiviral therapy (wk)	RT genotype	EC_{50} (μM) in CBMCs[a]			
			DXG	DAPD	3TC	AZT
3887	3TC (24)	184M/V	0.18	0.19	0.11	0.0007
4246	AZT (104)	wt	0.12	0.41	0.023	0.023
4526[b]	None	ND[c]	0.055	0.85	ND	0.0043
4877[d]	None	41L	0.045	0.26	ND	0.015
3350	3TC (12)	184V	0.65	3.3	>100	0.014
4205	3TC (52)	184V	1.1	4.1	>100	0.022
4242	AZT	41L 70R 215Y	0.21	0.88	0.009	0.15
4833[b]	Saquinavir (48)	ND	0.17	0.63	ND	0.062
4924[d]	AZT and nevirapine (26)	41L 103N	0.02	0.17	ND	0.001

[a] Values are the averages of data from two independent experiments conducted in duplicate.
[b] Neither RT nor protease genotypes were not determined. EC_{50}s for saquinavir were 0.0063 μM against isolate 4526 (baseline isolate) and 0.11 μM against isolate 4833.
[c] ND, not determined.
[d] EC_{50}s were obtained from a single experiment in duplicate. EC_{50}s for nevirapine were 0.065 μM against isolate 4877 (baseline isolate) and >10 μM against isolate 4924.

TABLE 6. Effects of combination of DXG with antiretroviral agents

Drug combination and molar ratio	CI* in CEMCs at inhibition level of:		
	EC_{50}	EC_{75}	EC_{90}
DXG and AZT			
10:1	0.61	0.27	0.12
20:1	0.57	0.29	0.15
40:1	0.67	0.30	0.14
DXG and 3TC			
1:1.6	0.79	0.59	0.47
1.25:1	0.82	0.58	0.46
2.5:1	0.74	0.52	0.42
DXG and nevirapine			
1.25:1	0.88	0.65	0.52
2.5:1	0.90	0.64	0.52
5:1	0.87	0.54	0.38

* CIs were analyzed by the method described by Chou and Talalay (5) and calculated by using CalcuSyn software (Biosoft, Cambridge, United Kingdom). CI values of 1, <1, and >1 indicate additive, synergistic, and antagonistic effects, respectively.

Our RT enzymatic analysis elucidated that DXG-TP, the putative active intracellular metabolite of DXG and DAPD, is a competitor of the natural substrate deoxyguanosine (Table 1) and a DNA synthesis chain terminator (data not shown). Similar to other nucleoside analogues (9, 11, 13), DXG-TP inhibits HIV-1 in vivo by competitively inhibiting the binding of the natural substrate dGTP to the HIV-1 RT. DXG-TP is incorporated into the nascent DNA strand, resulting in chain termination.

In summary, the dioxolanylpurine analogues DXG and DAPD are distinct from the related nucleoside analogue dideoxyguanosine in that they are selective inhibitors of the viral polymerase and have relatively low cellular toxicity, yielding a large selective index. These novel guanosine analogues have potent antiretroviral activity against both wild-type and drug-resistant HIV-1 variants, as well as synergistic activity when tested in combination with approved anti-HIV-1 agents. Thus, these observations suggest that the heterosubstituted guanosine analogues have potential as anti-HIV-1 drug candidates.

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